AngiomiR-126 expression and secretion from circulating CD34+ and CD14+ PBMCs: role for pro-angiogenic effects and alterations in type-2 diabetics

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Abstract: Several peripheral blood mononuclear cell (PBMC)-derived cell populations can promote angiogenesis, and differences in CD34+ or CD14+ surface-expression have been used to separate PBMC-subpopulations in this respect. AngiomiRs, microRNAs regulating angiogenesis, have been identified as key regulators of angiogenic processes. The present study examines differential angiomiR-expression/secretion from CD34+/CD14+; CD34+/CD14-; CD34-/CD14+; CD34-/CD14- PBMC-subsets and their relevance for different pro-angiogenic properties. Notably, both circulating human CD34+/14+ and CD34+/14- PBMC-subsets and their supernatants exerted more potent pro-angiogenic effects as compared to CD34- PBMC-subsets. MiR-126 was identified as most differentially expressed angiomiR in CD34+ as compared to CD34-PBMC-subsets, determined by miR-array and RT-PCR validation. Modulation of miR-126 by anti-miR-126 or miR-mimic-126 treatment resulted in significant loss or increase of pro-angiogenic effects of CD34+PBMCs. MiR-126 levels in supernatants of CD34+PBMC-subsets were substantially higher as compared to CD34- PBMC-subsets. MiR-126 was secreted in microvesicles/exosomes, and inhibition of their release impaired CD34+PBMCs pro-angiogenic effects. Notably, high-glucose treatment or diabetes reduced miR-126-levels of CD34+PBMCs, associated with impaired pro-angiogenic properties that could be rescued by miR-mimic-126 treatment. The present findings provide a novel molecular mechanism underlying increased pro-angiogenic effects of CD34+PBMC-subpopulations, i.e. angiomiR-126 expression/secretion. Moreover, an alteration of angiomiR-126-expression in CD34+PBMC in diabetes provides a new pathway causing impaired pro-angiogenic effects.

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AngiomiR-126 expression and secretion from circulating CD34+ and CD14+ PBMCs - role for pro-angiogenic effects and alterations in type-2 diabetics

Mocharla: microRNA126 in CD34+ cells and angiogenesis

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ABSTRACT

Several peripheral blood mononuclear cell (PBMC)-derived cell populations can promote angiogenesis, and differences in CD34+ or CD14+ surface-expression have been used to separate PBMC-subpopulations in this respect. AngiomiRs, microRNAs regulating angiogenesis, have been identified as key regulators of angiogenic processes. The present study examines differential angiomiR-expression/secretion from CD34+/CD14+; CD34+/CD14-; CD34-/CD14+; CD34-/CD14- PBMC-subsets and their relevance for different pro-angiogenic properties.

Notably, both circulating human CD34+/14+ and CD34+/14- PBMC-subsets and their supernatants exerted more potent pro-angiogenic effects as compared to CD34-PBMC-subsets. MiR-126 was identified as most differentially expressed angiomiR in CD34+ as compared to CD34-PBMC-subsets, determined by miR-array and RT-PCR validation. Modulation of miR-126 by anti-miR-126 or miR-mimic-126 treatment resulted in significant loss or increase of pro-angiogenic effects of CD34+PBMCs. MiR-126 levels in supernatants of CD34+PBMC-subsets were substantially higher as compared to CD34- PBMC-subsets. MiR-126 was secreted in microvesicles/exosomes, and inhibition of their release impaired CD34+PBMCs pro-angiogenic effects. Notably, high-glucose treatment or diabetes reduced miR-126-levels of CD34+PBMCs, associated with impaired pro-angiogenic properties that could be rescued by miR-mimic-126 treatment.

The present findings provide a novel molecular mechanism underlying increased pro-angiogenic effects of CD34+PBMC-subpopulations, i.e. angiomiR-126 expression/secretion. Moreover, an alteration of angiomiR-126-expression in CD34+PBMC in diabetes provides a new pathway causing impaired pro-angiogenic effects.

Key words: CD34+ cells, microRNA, angiogenesis, diabetes

Introduction

Several peripheral blood mononuclear cells (PBMC)-derived cell populations have been shown to promote angiogenesis and differences in both, CD34+ or CD14+ surface expression, have been used to separate functionally different PBMC sub-populations in this respect[1-5]. In particular, human CD34+ PBMC have been shown to promote angiogenesis in response to ischemia[1,3,5], resulting largely from paracrine effects. Administration of CD34+ PBMCs is also being evaluated as a potential treatment of ischemic heart disease and limb ischemia[6,7]. However, an impaired cardiovascular repair function of autologous patient-derived progenitor cells, in particular a reduced capacity to promote neo-vascularization, has been suggested in diabetes and ischemic heart disease[8-12], that may limit the efficacy of cell-based therapies in these patients and the underlying mechanisms need to be fully elucidated.

Moreover, PBMCs have been suggested as a source of both, early and late outgrowth "endothelial progenitor cells"[2] and differences in CD14+ expression have been proposed to distinguish mononuclear cells representing a source of early or late outgrowth cells in this respect[13]. The vast majority of early "endothelial progenitor cells" has been suggested to arise from CD14+ subpopulations of PBMCs, whereas late outgrowth "endothelial progenitor cells" with a potential for differentiation towards endothelial cells were reported to be derived from CD14- mononuclear cell fractions[2,13].

Importantly, several microRNAs (miRs) have recently been identified as critical regulators of vascular development and angiogenesis that have been termed angiomiRs[14-17]. Moreover detection of circulating miRs in serum/plasma as well as secretion of miRs or their release in apoptotic micro-particles has recently been reported, suggesting that miRs may also play a regulatory role outside of the cell and thereby impact on target cells[18-22]

The present study was therefore designed to determine whether there is differential angiomiR expression of these different PBMC subsets, separated for CD34+ expression as well as CD14+ surface expression, i.e. CD34+CD14+ and CD34+14- as well CD34-/14+ and CD34-14- PBMC subpopulations. Furthermore, we aimed to examine the potential role of differential angiomiR expression for differences in the pro-angiogenic capacity. In addition, the impact of high-glucose or type-2 diabetes on angiomiR expression and its relevance for the altered pro-angiogenic effects was examined.

Materials and Methods

Cell Isolation: PBMCs from healthy male volunteers (age: 49±4 years) were isolated from buffy coats by density-gradient centrifugation, washed twice in phosphate-buffered saline (PBS) containing 2 mM EDTA and 0.5% serum albumin. MNCs (~ 500x 10^6) were split, i.e. half of the PBMCs were incubated with anti-CD14 micro-beads (Miltenyi Biotec) for 30min., and separated through a column of a MACS separator (Miltenyi Biotec). The other half of PBMCs was incubated with Monocyte Isolation Kit II (Miltenyi Biotec) and isolated by negative selection according to the manufacturer's instructions, to obtain untouched CD14- (200-250 x10^6 cells) and CD14+ (100-150 x10^6 cells) fractions respectively. These fractions further underwent 2 cycles of anti-CD34+ micro-bead selection (Miltenyi Biotec), resulting in CD34+CD14+, CD34+ CD14-, CD34-CD14+, CD34-CD14- fractions of MNCs.

Anti-miR and miR-mimic-126 Transfection: CD34+ PBMCs were plated onto a 3cm dish over night and then harvested for electroporation. CD34+ cells (1x10^6) were transfected in 4mm cuvette with 25pmol of microRNA (scramble, anti-miR-126 (Exiqon) or miR-mimic-126 (Ambion) as indicated) in a total volume of 300µl. The conditions for electroporation were the following: 250V, 125 µF. Cells were incubated for 48 hours at 37°C and 5% CO_2.

MicroRNA RT PCR array: Total RNA was extracted from the fresh isolated cells using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. cDNA was generated from 1µg total RNA using the RT² microRNA First Strand Kit (SuperArray Bioscience) according to manufacturer's procedures. The Human Cell Differentiation & Development RT^2 microRNA PCR Array was carried out according to manufacturer's instructions. Real-time qRT–PCR was performed using the RT² qPCR SYBR Green/ROX Master Mix (SuperArray Bioscience) with pre-validated primer sets. Thermocycling parameters on a MX3000P PCR cycler (Stratagene) were 95°C for 10 min, followed by 40 cycles of 95°C for 15s, 60°C for 40s and 72°C for 30s. Samples from each cell population were run at least in triplicate and expression levels on each array plate were normalised using SNORD44 as internal control, according to the formula $NV = 2^{(CtSNORD44 - CtmiR)}$.

Validation MicroRNA RT PCR: Total RNA was extracted using QIAzol Reagent (Qiagen) according to the manufacturer's recommendations. 5ng of total RNA from each preparation was used for miRCURY LNA™ First-strand cDNA kit, in a final volume of 10µl, according to the manufacturer's instruction (Exiqon). Real time PCR was performed in an MX3000P PCR cycler (Stratagene). All experiments were performed in at least quadruplicate using the miRCURY LNA™ microRNA PCR system (Exiqon). Each reaction (25µl) contained 0.4 µl cDNA, 10 pmol of each primer, 0.25µl of internal reference dye, and 12.5µl of SYBR Green master mix. The

amplification program consisted of 1 cycle at 95 °C for 10min., followed by 40 cycles with a denaturing phase at 95 °C for 20s and an annealing phase at 60 °C for 1min. Data were normalised to results obtained with primers specific for U6 or in case of supernatants using synthetic spike-in miRNA (Exiqon), according to the formula $NV = 2^{(CtU6-CtmiR)}$.

Protein analysis: The cells were washed thrice with PBS and then fixed in 4% PFA. The cells were permeabilized with PBS/0.1% Triton X-100, and blocked in LI-COR buffer. The wells were then incubated with primary antibodies (1:200) in LI-COR blocking buffer (mouse anti-PI3KR2 antibody, abcam) overnight at 4°C. Infrared anti-mouse IRDye800CW secondary antibody (1: 200) and DRAQ5, highly cell permeable DNA-interactive agent, (1: 10,000) in PBS/0.5% Tween-20 were then added (50µl/well). The plates were imaged on Odyssey infrared scanner (LI-COR) using microplate2 settings with sensitivity of 4 in the 700 and 7 in the 800nm wavelength channels. Data were acquired by using Odyssey software (LI-COR), exported and analyzed in Excel (Microsoft).

Preparation of conditioned medium for functional and miR-analysis: Freshly isolated cell fractions, transfected cells and HAECs were cultured in EGM-2, 10% FCS (Lonza) incubated at 37°C and 5% CO_2. After overnight / 48hours incubation, cells were washed with PBS, re-suspended in EBM, 0.5%FCS (Lonza) and incubated for 12hours at 37°C and 5% CO_2.

***In-vitro* tube formation Assay:** Matrigel assays were performed as described previously [23] with slight modifications. Briefly, $2x10^3$ cells of CD34+CD14+, CD34+ CD14-, CD34-CD14+, CD34-CD14- PBMC fractions or transfected CD34+ cells were seeded onto 96-well tissue culture plates coated with 30µL Matrigel (BD Biosciences) and co cultured with $8x10^3$ human aortic endothelial cells (HAECs), resulting in a total cell density of $1x10^4$ cells/well. Cells were examined after 5hours with an inverted microscope (Olympus) at 40× magnification for capillary-like tube formation. Photographs were taken in 5 fields and complete tube numbers were counted.

Microvesicle (MV) and Exosome (Exo) Isolation from Cell Supernatants: The microvesicle and exosome fractions were isolated from cell supernatants as described previously[24]. In brief, $2x10^6$ cells from one buffy coat were incubated in serum free media which was centrifuged at 1000*g* for 10 min to remove cells. This supernatant was transferred to a new tube and centrifuged at 16K*g* for 60 min, the pellet (microvesicles) was washed and re-suspended in phosphate-buffered saline (PBS, 137mM Sodium Chloride, 2.7mM Potassium Chloride, 10mM Sodium Phosphate dibasic, 2mM Potassium Phosphate monobasic at pH of 7.4). The supernatant of the 16K*g* centrifugation was transferred to a new tube and further centrifuged at 120K*g* for 60min to pellet the exosomes. The exosome depleted supernatant was then

centrifuged at 220K*g* for 60min. The final supernatant was concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore) to a final volume of 0.5ml. The pellets, microvesicles, exosomes, and 220K x*g* pellet were re-suspended in 0.5ml PBS, so that the total volume of medium contributing to each was identical. For blocking of microvesicle/exosome release from CD34+ cells, these cells were treated with GW-4869 (10µM) for 30min as previously described[25] in EGM-2 (10% FCS).

Animals: Animal experiments were approved by the local committee. Male NRMI nu/nu mice, aged 10-16 weeks were used for transplantation of human PBMC subpopulations. Mice were housed under specific pathogen-reduced conditions receiving autoclaved chow and water ad libitum. For *in-vivo* angiogenesis assays in diabetic mice; 5 week old C57BL/6 mice were used. Diabetes was induced with streptozotocin in as described previously[26]. After 3 weeks of diabetes the animals were used for the experiments.

CD34+ Cell Isolation from Diabetic Patients: Written informed consent was obtained from all patients and healthy subjects, and the study protocol was approved by the local ethics committee. CD34+ PBMCs were also isolated from patients with type-2 diabetes and age-matched healthy subjects to compare the microRNA expression levels (57±3 vs. 53±5 years; P=n.s.).

***In-vivo* Angiogenesis assays:**

PBMC sub-fractions: *In-vivo* matrigel plug assay was performed as described previously[27] with the following modifications: 500µL of Matrigel Basement Membrane Matrix (BD Biosciences) containing 15 U of heparin (Sigma-Aldrich) was injected subcutaneously into 6- to 8-week-old female athymic nude mice along the abdominal midline. CD34+CD14+, CD34+ CD14-, CD34-CD14+, CD34-CD14- fractions were re-suspended in 400µL of PBS and injected via tail vein.

miR-mimic-126 administration: In C57BL/6 mice diabetic animals 100µL of Matrigel Basement Membrane Matrix (BD Biosciences) containing 15U of heparin (Sigma-Aldrich) was mixed with miR-mimic-126 and scrambled RNA (5, 15, 25 pmol each) and injected subcutaneously into the animals along the abdominal midline.

Microvesicle and exosome administration:

Increasing concentrations of the microvesicle and exosome fractions were mixed with 100µL of Matrigel Basement Membrane Matrix (BD Biosciences) containing 15U of heparin (Sigma-Aldrich) and injected subcutaneously into the animals along the abdominal midline.

Blood vessel quantification: After 4 days, blood vessel infiltration in Matrigel plugs was quantified by analysis of CD31-staining (BD pharmingen) with FITC-anti-rat antibody (AbD

Serotec). Sections were photographed (Nikon, Eclipse, TE300) at 100× magnification using NIS-Elements F3.0. For hemoglobin analysis, the Matrigel plug was removed after 4 days and homogenized in 100µL of de-ionized water. After centrifugation, the supernatant was used in the Drabkin assay (Sigma-Aldrich) to measure hemoglobin concentration. Stock solutions of hemoglobin are used to generate a standard curve. Results are expressed relative to plug weight.

Statistical Analysis: Quantitative results are expressed as means ± SEM. Comparisons between groups were analysed using one-way ANOVA, followed by Bonferroni post-hoc test. A value of $P<0.05$ was considered to be statistically significant.

Results

Both CD34+CD14+ and CD34+CD14- PBMC subsets exhibit a substantially greater pro-angiogenic capacity as compared to CD34-/CD14+ and CD34-/CD14- MNC subsets *in-vitro* and *in-vivo*

Tube formation capacity was compared for two CD34+ PBMC subsets (CD34+CD14+ and CD34+CD14-) and two CD34- cell PBMC subsets (CD34-CD14+ and CD34-CD14-) isolated from circulating PBMCs. Both CD34+ cell fractions had a substantially higher pro-angiogenic capacity as indicated by an increased tube formation capacity in HAEC-co-culture assay, as compared to CD34-CD14+ and CD34-CD14- PBMC fractions (Fig.1A,B). Since CD34+ PBMCs have been suggested to promote angiogenesis largely via paracrine mechanisms, conditioned medium from the CD34+ and CD34- PBMC fractions was assessed. Supernatants from CD34+ PBMC fractions had a higher stimulatory capacity for tube formation as compared to CD34- PBMC-fraction supernatants. No significant difference was observed between CD34+CD14+ and CD34+CD14- PBMC supernatants (Fig.1C). The analysis of the pro-angiogenic capacity *in-vivo,* in line with the above results, suggested a higher pro-angiogenic stimulatory capacity of both CD34+ PBMC fractions as compared to CD34- PBMC fractions (Fig.1D). Furthermore, we observed a more pronounced effect of CD34+ cells on angiogenesis *in-vivo* using the matrigel plugs with an increasing CD34+ cell number (Suppl. Fig. 3A).

Increased angiomiR miR-126 expression in CD34+ as compared to CD34- PBMC subsets

To investigate potential novel mechanisms explaining different pro-angiogenic effects in CD34+ and CD34- PBMC subpopulations, angiomiR expression patterns were assessed in the above cell populations. Human Cell Differentiation & Development RT PCR array was performed using total RNA isolated from CD34+CD14+, CD34+ CD14-, CD34-CD14+, CD34-CD14- cell fractions. Within the 8 most expressed miRs of the microRNA array we identified the angiomiR miR-126 (Fig.2A i-viii). Moreover, the miR-126 was identified as the angiomiR that was most profoundly higher expressed in CD34+ as compared to CD34- MNC sub-fractions (Fig.2B i-viii). The expression profile of other angiomiRs detected in the array and validated are shown in Fig.3A,B. Of note, CD34-CD14+ PBMCs had also higher expression levels of potentially anti-angiogenic miRs, such as miRs from the miR-17-92 cluster or miR-21 (Fig.3B).

Pro-angiogenic capacity of CD34+ PBMCs is dependent on miR-126 expression

MiR-mimic-126 or anti-miR-126 treatment resulted in a markedly altered pro-angiogenic capacity of the CD34+, i.e. an enhanced tube formation was seen after miR-mimic-126 transfection into CD34+ cells, whereas a decreased tube formation was observed in response to anti-miR-126 transfected CD34+ cells (Fig.4A,B). Similar results were observed when tube formation was examined in response to conditioned medium from CD34+ cells that were transfected with miR-mimic-126 or anti-miR-126, suggesting that the pro-angiogenic capacity of CD34+ cells is dependent on miR-126 and is largely mediated via paracrine factors (Fig.4C,D). CD34+ cells transfected with anti-miR-126 or miR-mimic-126 showed altered levels of miR-126, i.e. reduced or increased miR-126 levels, respectively (Fig.4E).

MiR-126 is secreted from CD34+ cells largely in microvesicles/exosomes and stimulates angiogenesis

MiR-126 levels were determined in supernatants from CD34+CD14+, CD34+CD14-, CD34-CD14+, CD34-CD14- MNC populations, and observed significantly higher secretion of miR-126 in supernatants of CD34+ cell populations. No significant difference was observed between the CD34+CD14+ and CD34+CD14- cell fractions (Fig.5A). Modulation of the expression of miR-126 by anti-miR or miR-mimic-126 in CD34+ cells leads to a decrease or increase in the secretion of this angiomiR into supernatants, respectively (Fig.5B). MiR-126 was largely detected in microvesicles and to a lesser extent in exosomes (Fig.5C). Administration of microvesicles or exosomes derived from CD34+ cells to endothelial cells resulted in a stimulation of tube formation (Fig.4D). Microvesicles or exosomes stimulated angiogenesis in the matrigel plug assay *in-vivo* and increasing concentrations of mirovesicles had a greater effect on *in-vivo* angiogenesis (Suppl. Fig.1A,B). We further examined the effects of microvesicles or exosomes derived from antimiR-126-transfected and miR-mimic-126 transfected CD34+ cells on angiogenesis in the matrigel plug assay *in-vivo.* Microvesicles or exosomes from miR-126 deficient CD34+ cells had a substantially reduced effect on *in-vivo* angiogenesis (Suppl. Fig.2A,C), whereas microvesicels and exosomes derived from miR-mimic-126 transfected cells had an enhanced effect on *in-vivo* angiogenesis (Suppl. Fig.2B,D).

The transfer of secreted vesicles from CD34+ cells to the endothelial cells was assessed by adding the supernatants from PKH67 stained CD34+ cells. The vesicular fusion into endothelial cells resulted in acquisition of the color after overnight incubation. The majority of endothelial cells acquired the color as assessed by flow-cytometry analysis (Fig.5E).

Blocking of the secretion of the vesicular particles by using GW4869 resulted in reduction of secreted miR-126 in the supernatant of the treated CD34+ cell and a subsequent increase of the miR in CD34+ cells (Fig.5F,G). GW4869 is an inhibitor of neutral sphingomyelinase 2 that regulates ceramide synthesis which is crucial for triggering the secretion of membrane vesicles[25] Treatment of CD34+ cells with GW4869 lead to a loss of pro-angiogenic stimulation of the conditioned medium derived from these cells as compared to untreated CD34+ cells (Fig.5H). Moreover, we have also examined the effects of different doses of the miR-mimic-126 or scrambled-miR on *in-vivo* angiogenesis. In these experiments, the higher doses of the miR-mimic-126, but not of the scrambled-miR, had a more pronounced effect on *in-vivo* angiogenesis (Suppl. Fig.3B,C).

High glucose treatment or diabetes leads to a loss of miR-126 expression and pro-angiogenic effects of CD34+ PBMCs that can be restored by miR-mimic-126 transfection

Exposure of CD34+ PBMCs to high-glucose substantially decreased their subsequent proangiogenic capacity as determined after washing of CD34+ PBMCs and subsequent co-culture with HAECs, i.e. using an *in-vitro* tube formation assays (Fig.6A,B). This loss of function was likely due to an alteration of secreted factors since decreased tube formation in HAECs was observed after treatment with the conditioned medium from high-glucose treated CD34+ PBMCs (Fig.6C,D). Notably, expression of the pro-angiogenic miR-126 was down-regulated in high-glucose-treated CD34+ PBMCs as compared with buffer-treated cells in both, CD34+ CD34+ PBMCs and their supernatants (Fig.6E). Of-note, CD34+ PBMCs derived from patients with type-2 diabetes had a substantially decreased miR-126 expression as compared with age and sex-matched healthy subjects (Fig.6F).

Furthermore, CD34+ PBMCs exposed to high-glucose were transfected with miR-mimic-126 or scrambled miR and their capacity to promote endothelial tube formation was subsequently assessed. Notably, miR-mimic-126 treated high-glucose exposed CD34+ PBMCs showed a marked improvement in the promotion of tube formation (Fig.7A,B).

Direct exposure of endothelial cells to miR-mimic-126 stimulates angiogenesis *in-vitro* and *in-vivo*

In addition, to determine whether miR-mimic-126 exposure of endothelial cells could directly impact angiogenesis, both *in-vitro* and *in-vivo* administration of miR-mimic-126 was examined. Notably, as described above we have observed that miR-126 is released from CD34+ cells. Moreover, CD34+ cell supernatants increased miR-126 levels in high-glucose-treated endothelial cells (Fig 7 C). MiR-mimic-126 exposure, but not scrambled-miR administration to

high-glucose-treated endothelial cells significantly increased their miR-126 levels (data not shown). Notably, addition of the miR-mimic-126 directly to the medium of high-glucose exposed HAECs increased tube formation as compared to addition of scrambled-miR (Fig.7D) and down-regulated PI3KR2, a known target of miR-126 in endothelial cells (Fig.7E). Moreover, addition of miR-mimic-126, but not scrambled miR directly to the matrigel plug implanted in streptozotocin-induced diabetic animals enhanced tube formation and increased hemoglobin content (as a sign of increased new vessel formation) in the matrigel plug as compared with scrambled miR-treated animals (Fig.7F,G). These findings suggest that direct exposure of miR-mimic-126 can promote these angiogenic processes.

Discussion

In the present study we have observed a substantially higher expression of the angiomiR miR-126 in human CD34+ as compared to CD34- PBMC subsets. Notably, miR-126 was secreted by CD34+ PBMC subsets, largely in microvesicles and exosomes, and this secretion was altered by miR-mimic-126 or anti-miR-126 treatment. Modulation of miR-126 expression levels by either miR-mimic-126 or anti-miR-126 revealed that miR-126 is critical for the pro-angiogenic capacity of CD34+ PBMCs. In addition, we observed that reduced miR-126 expression is a novel mechanism leading to an impaired pro-angiogenic capacity in high-glucose-treated or diabetic patient-derived CD34+ PBMCs, which could be reversed by miR-mimic-126 treatment.

Previous studies have suggested that at least 2 morphologically and functionally distinct "endothelial progenitor cell" populations can be derived after culture from circulating mononuclear cells according to duration of culture and the baseline CD14 phenotypic marker expression[2,28]. We have observed that the CD34+ fraction of cells showed a more potent pro-angiogenic effect *in-vitro* and *in-vivo*, however, we did not observe a significant difference between freshly isolated CD34+CD14+ and CD34+CD14- MNC-derived cell populations with respect to their pro-angiogenic capacity. Previous studies have demonstrated that monocytes can stimulate angiogenesis [2,4,28,29]. With the results of the present study one may speculate that the CD34+CD14+ MNC cell fraction may in particular contribute to such pro-angiogenic effects. We have observed that the CD34+ CD14+ MNC fraction resulted in a significant promotion of tube formation, very similar to that observed in the CD34+ CD14- MNC fraction.

Notably, the present study for the first time demonstrates different angiomiR expression profiles in the PBMC sub-populations separated for their CD34+ and CD14+ surface expression. MiR-126 was substantially increased in both, the CD34+CD14+ and CD34+CD14- PBMC populations and was critical for the increased pro-angiogenic capacity of the CD34+ PBMC population. Previously, miR-126 has been suggested to play a critical role in vascular development[30,31]. Moreover, following myocardial infarction miR-126 knockout mice showed impaired vascular growth in the infarct border zone[30,31]. A reduced expression of potentially anti-angiogenic miRs in CD34+ cells as compared to CD34- cells, such as miR-15a or miR-20a levels observed in the present study, may also support an increased pro-angiogenic capacity of CD34+ cells. However, given the observation that anti-miR-126 treated CD34+ cells had lost the capacity to stimulate angiogenesis, these differentially expressed miRs are likely not sufficient to explain the functional difference observed between CD34+ and CD34- MNC cell subsets.

Moreover, in the present study we have observed that the pro-angiogenic miR-126 is enriched in microvesicles and exosomes secreted by CD34+ progenitor cells and that these vesicles are

taken-up by endothelial cells. The increased miR-126 levels in vesicles correlated with an increased tube formation capacity. The presence of growth factors or other pro-angiogenic factors inside the vesicles [20], together with miR-126 likely mediates pro-angiogenic effects associated with these vesicles. The molecular content transported by CD34+ exosomes still remains to be fully characterised. Notably, exosomes with reduced miR-126 content did not stimulate the tube formation of endothelial cells; raising the possibility that miR-126 is a paracrine factor stimulating endothelial cell angiogenic activity. Zernecke et al have recently suggested that miR-126 is released from endothelial apoptotic micro-particles and acts in a paracrine way[22]. These micro-particles were incorporated by surrounding endothelial cells, and miR-126 transfer has been proposed to increase production of CXCL12 in endothelial cells, promoting homing of progenitor cells after vascular injury.

In the present study we have observed that protein levels of phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2), a known target of miR-126, are reduced in endothelial cells upon administration of miR-mimic-126, suggesting that the microRNA uptake regulates target expression in endothelial cells. PIK3R2 has been shown to inhibit pro-angiogenic signalling in endothelial cells by suppression of growth factor signalling via PI3 kinase pathways.[30,31]

Diabetes has been observed to lead to an impaired function of early endothelial progenitor cells[8,9,12]. In the present study we suggest a novel link between loss of pro-angiogenic stimulation and reduced miR-126 levels in CD34+ cells in high-glucose treated cells. Plasma microRNA profiling in patients with type-2 diabetes has recently revealed reduced plasma levels of miR-126 in diabetic patients[32]. The present study newly demonstrates a reduced miR-126 release from CD34+ PBMCs after high-glucose exposure and in diabetic patients that could contribute to reduced circulating miR-126 levels.

The present observations do not exclude that other microRNAs beyond miR-126 may also regulate the angiogenic capacity of CD34+ cells as well that needs to be further explored in future studies. For microRNA-126 we have shown that it is clearly promoting the pro-angiogenic capacity of CD34+ cells. Others have reported that miR-126-knockouts had an impaired angiogenic response in the heart in the setting of myocardial ischemia and promote endothelial cell tube formation[30]. At the same time, miR-126 has very recently been reported to suppress vascular growth in breast cancer metastasis by suppressing endothelial cell recruitment to metastatic cells via repression of other targets expressed in metastatic breast cancer cells[33], suggesting that miR-126 does not promote tumor-associated angiogenesis. The effects of microRNA on angiogenesis may therefore substantially differ in diverse cell types and patho-physiological settings.

Two other microRNAs that were found to be higher expressed in CD34+ cells as compared to CD34- mononuclear cells in the present study have very recently been suggested to be involved in the regulation of angiogenic processes in different endothelial cell types. We observed miR-100 expression to be higher in CD34+ as compared to CD34- mononuclear cells. MiR-100 expression in endothelial cells has been suggested to inhibit proliferation, tube formation, and sprouting activity and to act as an endogenous repressor of the serine/threonine protein kinase mammalian target of rapamycin[34]. This raises the possibility that this microRNA would rather limit the pro-angiogenic capacity of CD34+ mononuclear cells that needs, however, to be evaluated in future studies. MiR-10b was higher expressed in CD34+ as compared to CD34- mononuclear cells in the present study. MiR-10b has recently been suggested to regulate angiogenesis in human micro-vascular endothelial cells, where miR-10b over-expression promoted endothelial cell migration and tube formation[35]. These findings raise the possibility that this microRNA may also regulate pro-angiogenic effects in CD34+ mononuclear cells, that needs to be further evaluated in future studies. However, miR-10b was substantially higher expressed in CD34+CD14+ as compared to CD34+CD14- mononuclear cells, that had a rather similar pro-angiogenic capacity in the present study, that would suggest that miR-10b is not a major regulator of pro-angiogenic effects of CD34+ mononuclear cells. Vice versa, it is also possible that higher expression of potentially anti-angiogenic microRNA in CD34- mononuclear cells, such as from the miR-17-92 cluster or miR-21 as observed in the present study, may limit the pro-angiogenic capacity of CD34- mononuclear cells.

In summary, our present findings demonstrate a differential angiomiR expression in CD34+ and CD34- PBMC subpopulations. Increased angiomiR miR-126 expression in CD34+ PBMCs as compared to CD34- PBMCs was identified as a novel mechanism contributing to the increased capacity of these cells to promote angiogenic processes. Moreover, down-regulation of miR-126 in CD34+ PBMCs after high-glucose exposure or in diabetic patients represents a novel mechanism leading to an altered pro-angiogenic capacity in these conditions that can be rescued by miR-mimic-126 transfer.

Authorship contribution

P M: designed and performed research, analyzed and interpreted, performed statistical analysis data and wrote the manuscript

S B: performed the experiments, analyzed and interpreted data, performed statistical analysis, and revised the manuscript

GG: performed the experiments and revised the manuscript

CD: performed the animal experiments and revised the manuscript

PJ: performed the animal experiments and revised the manuscript

FP: performed the animal experiments and revised the manuscript

TL: contributed to planning of experiments and revised the manuscript

UL: conceived and designed the experiments and wrote the manuscript

All authors declare no competing financial interests

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Abbreviations

AngiomiR- Angiogenic microRNA

CD- Cluster of differentiation

ECs- Endothelial cells

Exo- exosomes

HAECs- Human aortic endothelial cells

HG- High glucose

miR- MicroRNA

MNC- Mononuclear cells

MV- Microvesicles

NG- Normal glucose

PBMCs- Peripheral blood mononuclear cells

Figure Legends

Fig.1: CD34+CD14+ and CD34+CD14-+ PBMCs exhibit increased pro-angiogenic effects as compared to CD34-CD14+ and CD34-CD14- PBMCs.

A: Quantification of tube formation by CD34+CD14+ and CD34+CD14- (white and black) as opposed to CD34-CD14+ and CD34-CD14- (grey), B: representative photographs of tube formation in high power field (n=8). C: conditioned medium of CD34+ fraction of PBMCs (white and black) promoting tube formation, which was not observed in the CD34- PBMC fractions (grey) (n=5-6) D: CD31 staining quantification by image j, in sections of *in-vivo* matrigel plug sections (n=4), ECs= endothelial cells. Data are mean ± SEM.

Fig.2: MicroRNA expression profiles in PBMCs separated for CD34+ and CD14+ surface expression.

A: The 8 most abundant miRs detected using this array are shown. B: Moreover, 8 miRs were identified that were significantly higher expressed in CD34+ PBMCs as compared to CD34- PBMC subsets, of which miR-126 was the most abundant. $CD34^+CD14^+$ (white), $CD34^+CD14^-$ (black), $CD34^-CD14^+$ and $CD34^-CD14^-$ (grey). All miRNAs were normalized to SNORD6 and expressed as normalized values (NV). Data are mean ± SEM.

Fig.3: Expression of pro-angiogenic angiomiR miR-126 is higher in CD34+ PBMC sub-populations.

A: Confirmation of microRNA-array results and expression of other potentially pro-angiogenic angiomiR as determined by RT-PCR (n=6-8). B: Expression levels of other potentially anti-angiogenic miR in the different MNC subpopulations (n=6-8). All angiomiRs expression is normalized to U6 small nuclear RNA and expressed as normalized value (NV). U6 levels were not altered in the different MNC subpopulations (data not shown). Data are mean ± SEM.

Fig.4: Increased pro-angiogenic capacity of CD34+ PBMCs is critically dependent on miR-126 expression.

A: Shows transfected CD34+ cells, with antimiR-126 (grey) and miR-mimic 126 (black), effect on tube formation stimulation respectively as compared with that of scrambled (white) transfected cells. B: Representative photographs of tube formation in high power field. (n=6-10) C: Supernatant of transfected CD34+ cells effect on pro-angiogenic stimulation of endothelial cells is shown D: Photographs of tube formation in high power field (n=4). E: Altered miR-126 expression levels upon transfection of CD34+ cells with

anti-miR-126 (grey) and miR-mimic-126 (black). (n=4-6) MicroRNA expression is normalized to U6, small nuclear RNA and expressed as normalized value (NV). Data are mean ± SEM.

Fig.5: miR-126 is secreted by CD34+ PBMCs in microvesicular and exosomal fractions.

A: Assessment of miR-126 levels in the supernatant of the different populations of blood MNCs: CD34+CD14+ (white), CD34+CD14- (black), CD34-CD14- cells (grey bars) (n=3-5). B: miR-mimic-126 or antimiR-126 treatment leads to increased (black) or reduced (white) miR-126 levels in the supernatant respectively (n=4). C: Expression levels of miR-126 in various fractions of the supernatant (n=4). D: pro-angiogenic stimulation by microvesicles (white), exosomes (grey) and 220k pellet (black) (n= 4-6). E: Internalisation of vesiclesby endothelial cells, detected in FACS analysis by acquisition of PKH-67 stain (n=3). F-H: miR-126 expression levels in cells and supernatants of CD34+ cells treated with GW4869 and effect on tube formation (n=4). Data are mean ± SEM.

Fig.6: High glucose exposure/ diabetes leads to loss of pro-angiogenic effects and miR-126 expression and release from CD34+ PBMCs.

A: Effect of high glucose 25mM (black) treatment as compared with the normal glucose treated CD34+ cells (n=5-10). B: Representative photographs of tube formation in high power field. C: Supernatant of high glucose (black) treated cells as opposed to normal glucose (white) their effect on tube formation (n= 6-8). D: Photographs of tube formation in high power field. E: Assessment of miR-126 in high glucose treated healthy CD34+ cells compared to untreated CD34+ cells (n=4). F: Assessment of the miR-126 levels in patients with type-2 diabetes (black) CD34+ cells in comparison with healthy subjects (white) (n=3). HG- High glucose; NG- Normal glucose. Data are mean ± SEM.

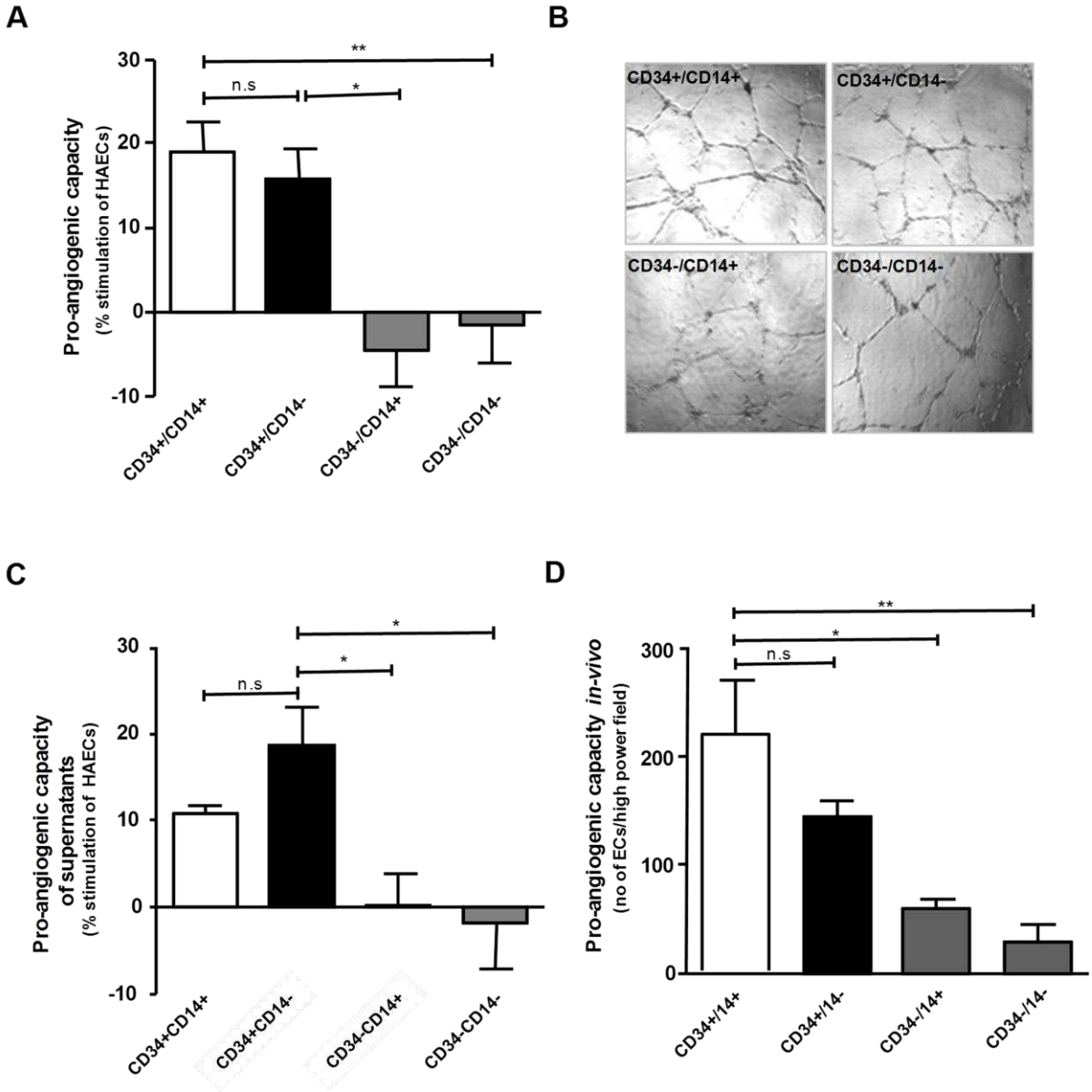
Fig.7: Pro-angiogenic capacity of CD34+ cells and capillary formation is increased by increasing miR-126 levels in high glucose-treated CD34+ cells.

A: High glucose treated CD34+ cells were transfected with scrambled (white) and miR-mimic-126 (black) and the effects on endothelial cell tube formation are shown. B: Representative photograhs of tube formation in high power field. (n=4) C: MiR-126 levels in high-glucose-treated (HG) endothelial cells after treatment with healthy CD34+ cell supernatant. D: Effect of direct exposure of high-glucose-treated HAECs to 25pmol scrambled-miR (white) or miR-mimic-126 (black) on tube-formation is shown. n=5. E: Expression levels of PI3KR2 in high-glucose-treated HAECs treated with 25pmol scrambled-miR (white) or miR-mimic-126 (black). (n=3-5) F: Capillaries in the plug were

assessed by staining for CD31 (green) and DAPI (blue) in *in-vivo* matrigel plug assay in diabetic mice (streptozotocin-induced) after administration of miR-mimic-126 or scrambled miR – a representative image is shown. G: Hemoglobin content was assessed in the subcutaneously implanted matrigel plugs of animals receiving scrambled or miR-mimic-126 (directly into plugs) (n= 4). Data are mean ± SEM.

Figures

Figure : 1



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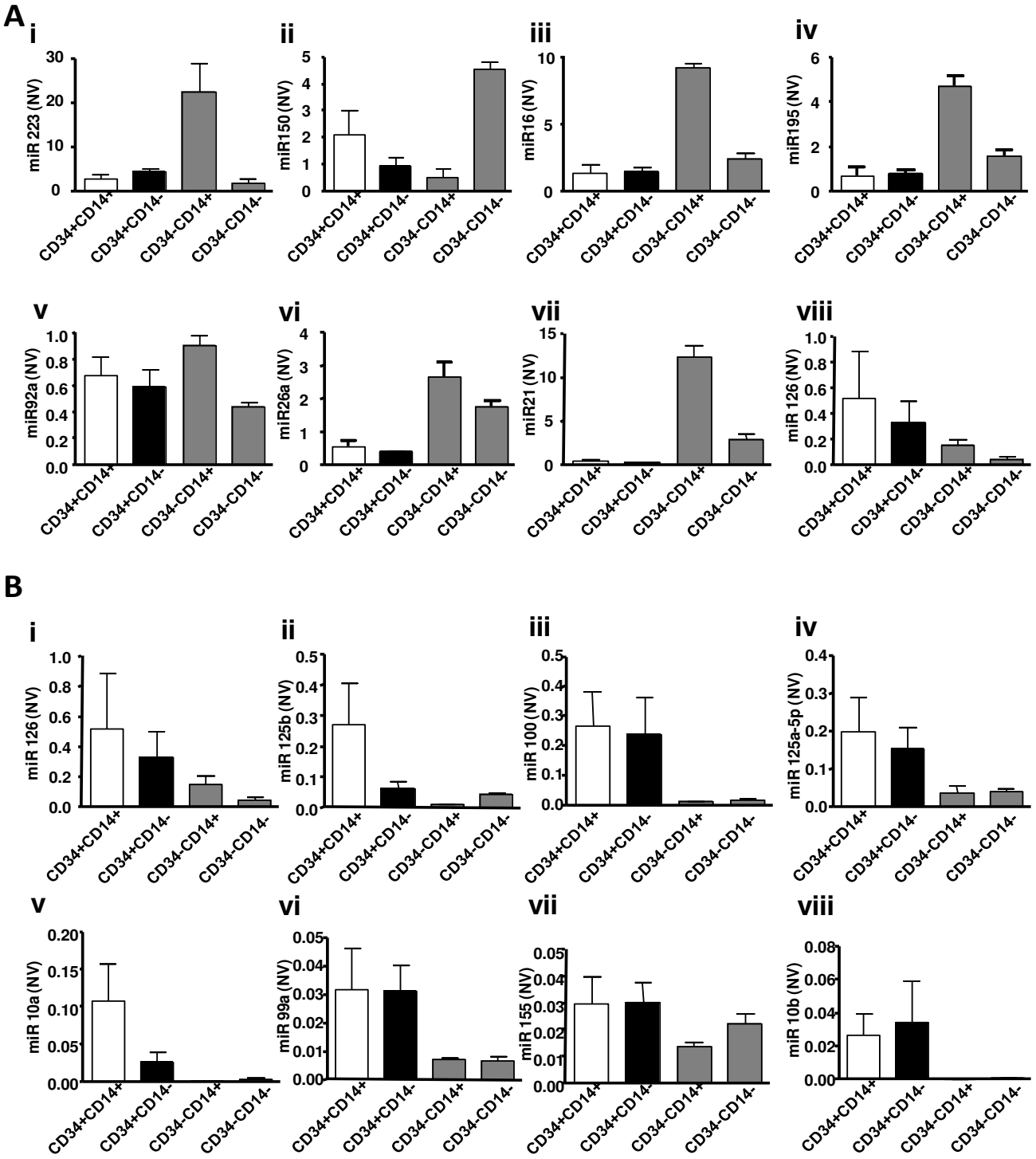

A
i
miR 223 (NV)
ii
miR150 (NV)
iii
miR16 (NV)
iv
miR195 (NV)
v
miR92a (NV)
vi
miR26a (NV)
vii
miR21 (NV)
viii
miR 126 (NV)
CD34+CD14+
CD34+CD14-
CD34-CD14+
CD34-CD14-
B
i
miR 126 (NV)
ii
miR 125b (NV)
iii
miR 100 (NV)
iv
miR 125a-5p (NV)
v
miR 10a (NV)
vi
miR 99a (NV)
vii
miR 155 (NV)
viii
miR 10b (NV)

Figure : 3

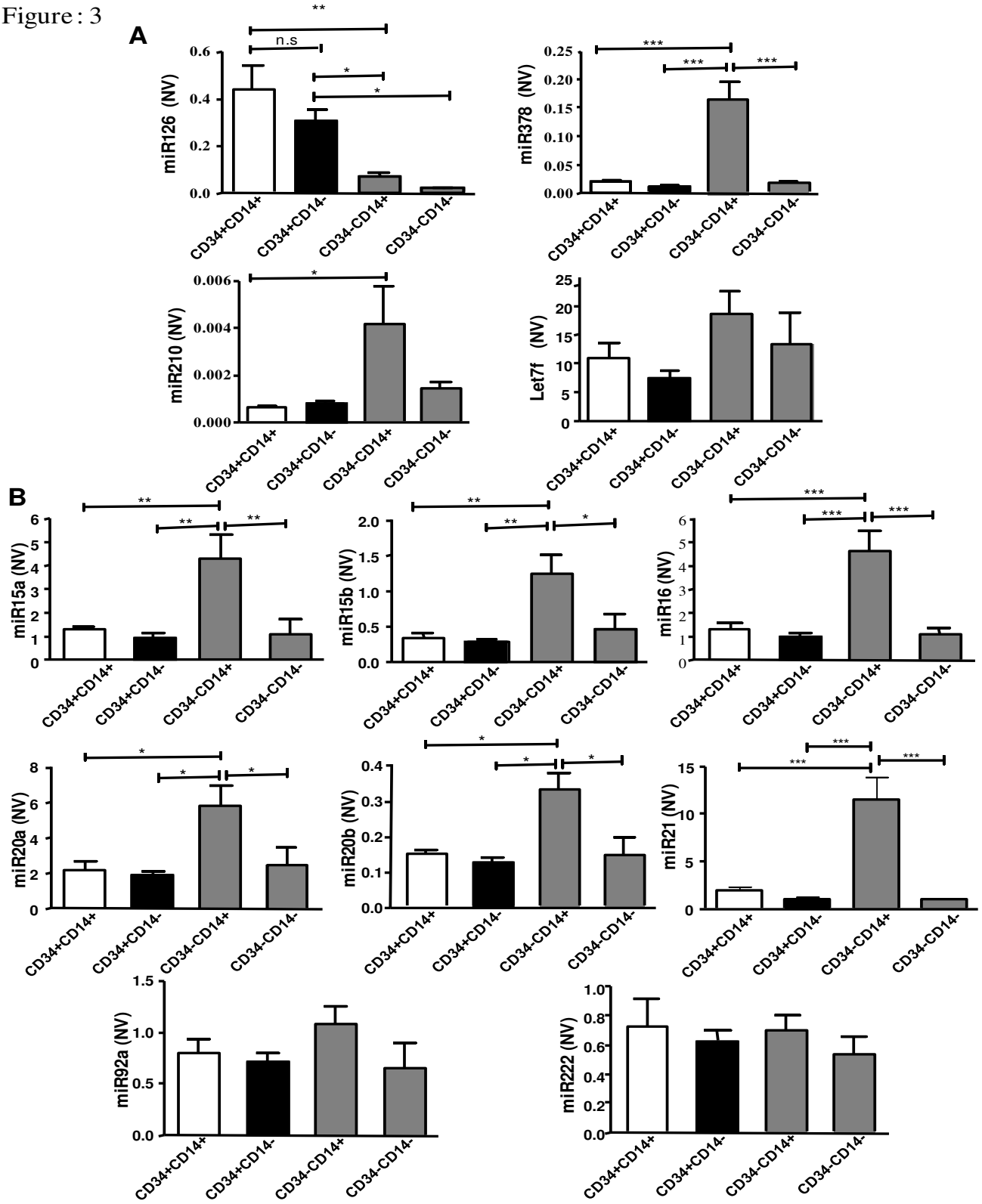

A
miR126 (NV)
miR378 (NV)
miR210 (NV)
Let7f (NV)
B
miR15a (NV)
miR15b (NV)
miR16 (NV)
miR20a (NV)
miR20b (NV)
miR21 (NV)
miR92a (NV)
miR222 (NV)
CD34+CD14+
CD34+CD14-
CD34-CD14+
CD34-CD14-
n.s

Figure : 4

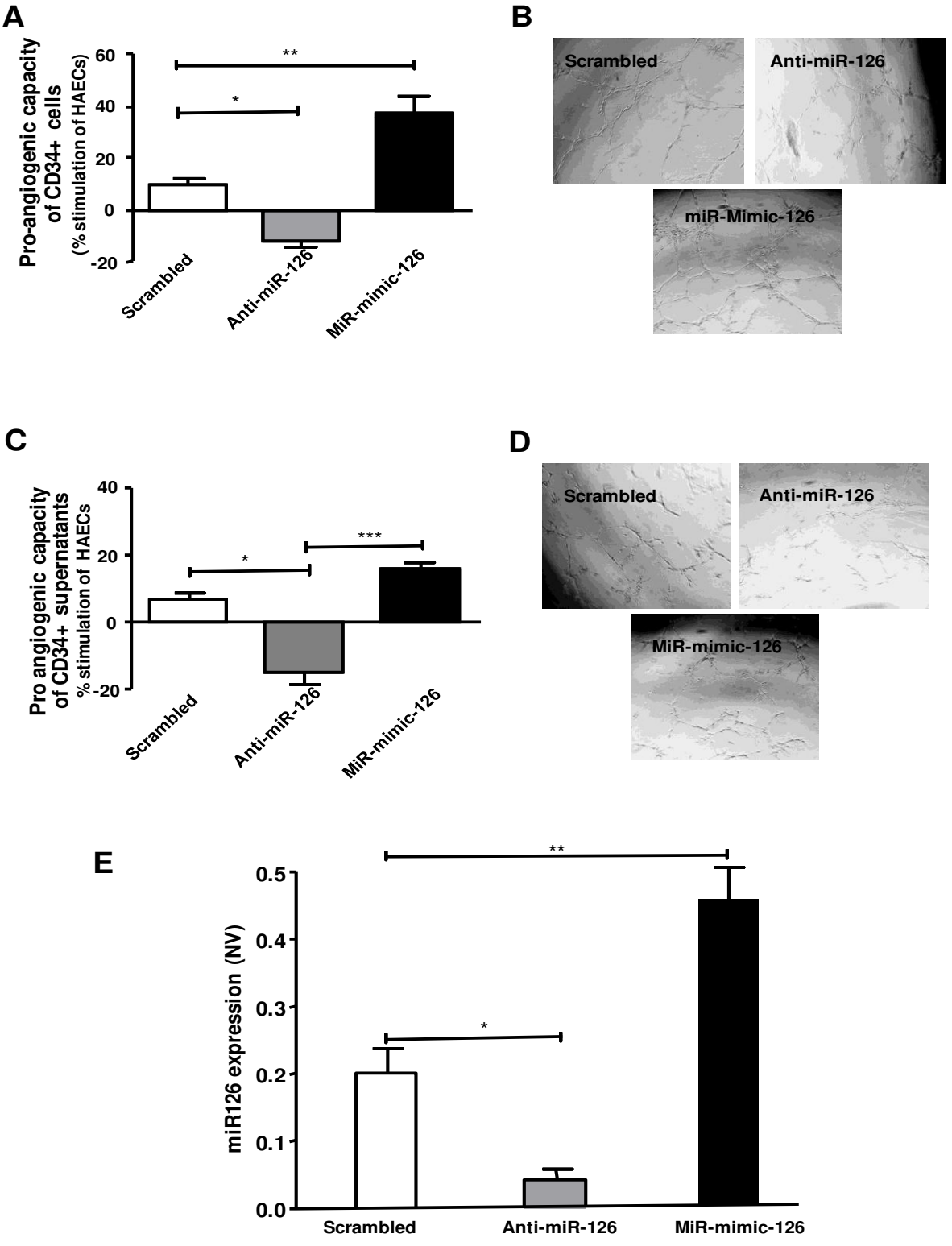

A
Pro-angiogenic capacity of CD34+ cells (% stimulation of HAECs)
60
40
20
0
-20
**
*
Scrambled
Anti-miR-126
MiR-mimic-126
B
Scrambled
Anti-miR-126
miR-Mimic-126
C
Pro angiogenic capacity of CD34+ supernatants % stimulation of HAECs
40
20
0
-20
*
***
Scrambled
Anti-miR-126
MiR-mimic-126
D
Scrambled
Anti-miR-126
MiR-mimic-126
E
miR126 expression (NV)
0.5
0.4
0.3
0.2
0.1
0.0
**
*
Scrambled
Anti-miR-126
MiR-mimic-126

Figure : 5

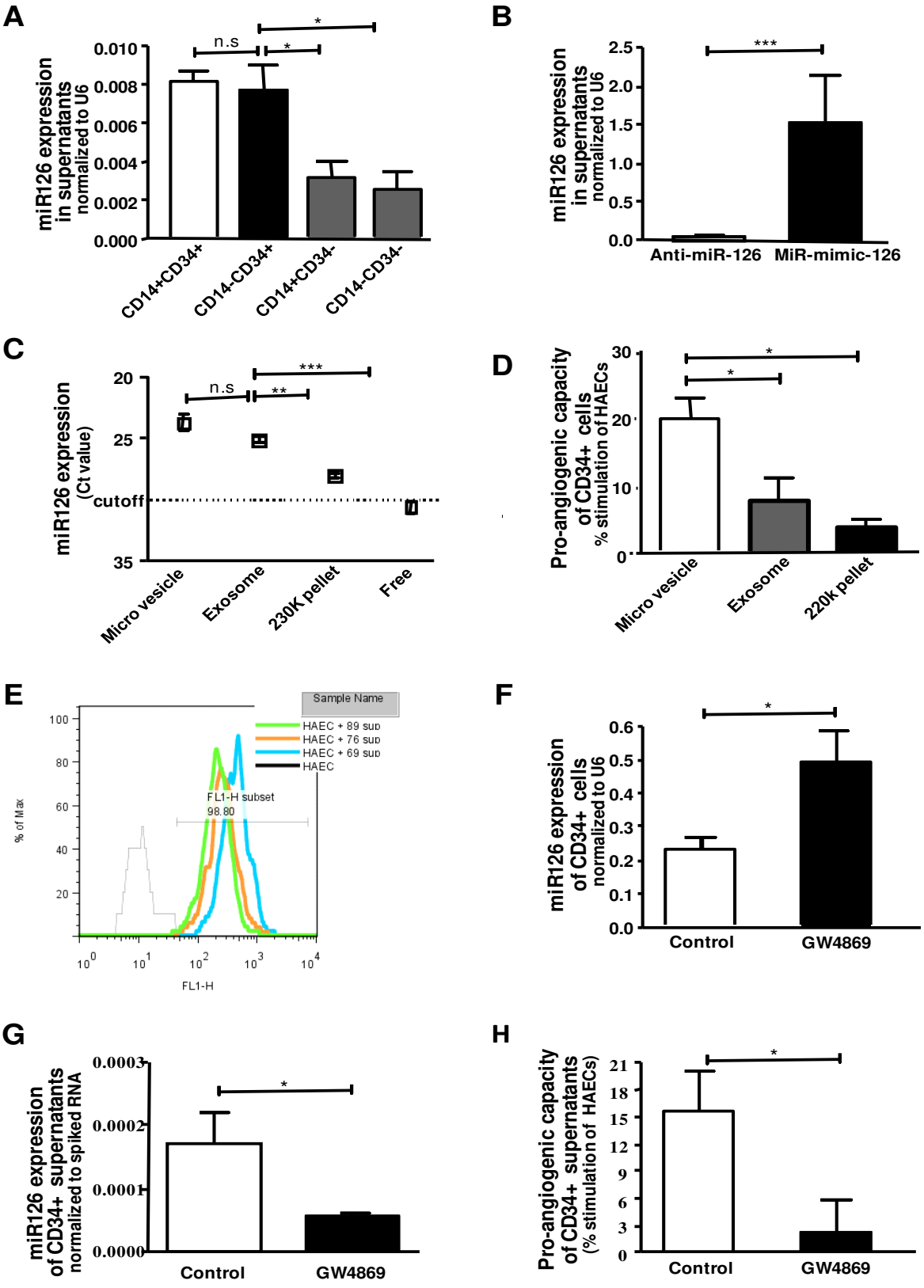

A
miR126 expression in supernatants normalized to U6
0.010
0.008
0.006
0.004
0.002
0.000
n.s
*
*
CD14+CD34+
CD14-CD34+
CD14+CD34-
CD14-CD34-
B
miR126 expression in supernatants normalized to U6
2.5
2.0
1.5
1.0
0.5
0.0
***
Anti-miR-126
MiR-mimic-126
C
miR126 expression (Ct value)
20
25
cutoff
35
n.s
**
***
Micro vesicle
Exosome
230K pellet
Free
D
Pro-angiogenic capacity of CD34+ cells % stimulation of HAECs
30
20
10
0
*
*
Micro vesicle
Exosome
220k pellet
E
Sample Name
HAEC + 89 sup
HAEC + 76 sup
HAEC + 69 sup
HAEC
FL1-H subset
98.80
% of Max
100
80
60
40
20
10^0
10^1
10^2
10^3
10^4
FL1-H
F
miR126 expression of CD34+ cells normalized to U6
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
GW4869
G
miR126 expression of CD34+ supernatants normalized to spiked RNA
0.0003
0.0002
0.0001
0.0000
*
Control
GW4869
H
Pro-angiogenic capacity of CD34+ supernatants (% stimulation of HAECs)
21
18
15
12
9
6
3
0
*
Control
GW4869

Figure : 6

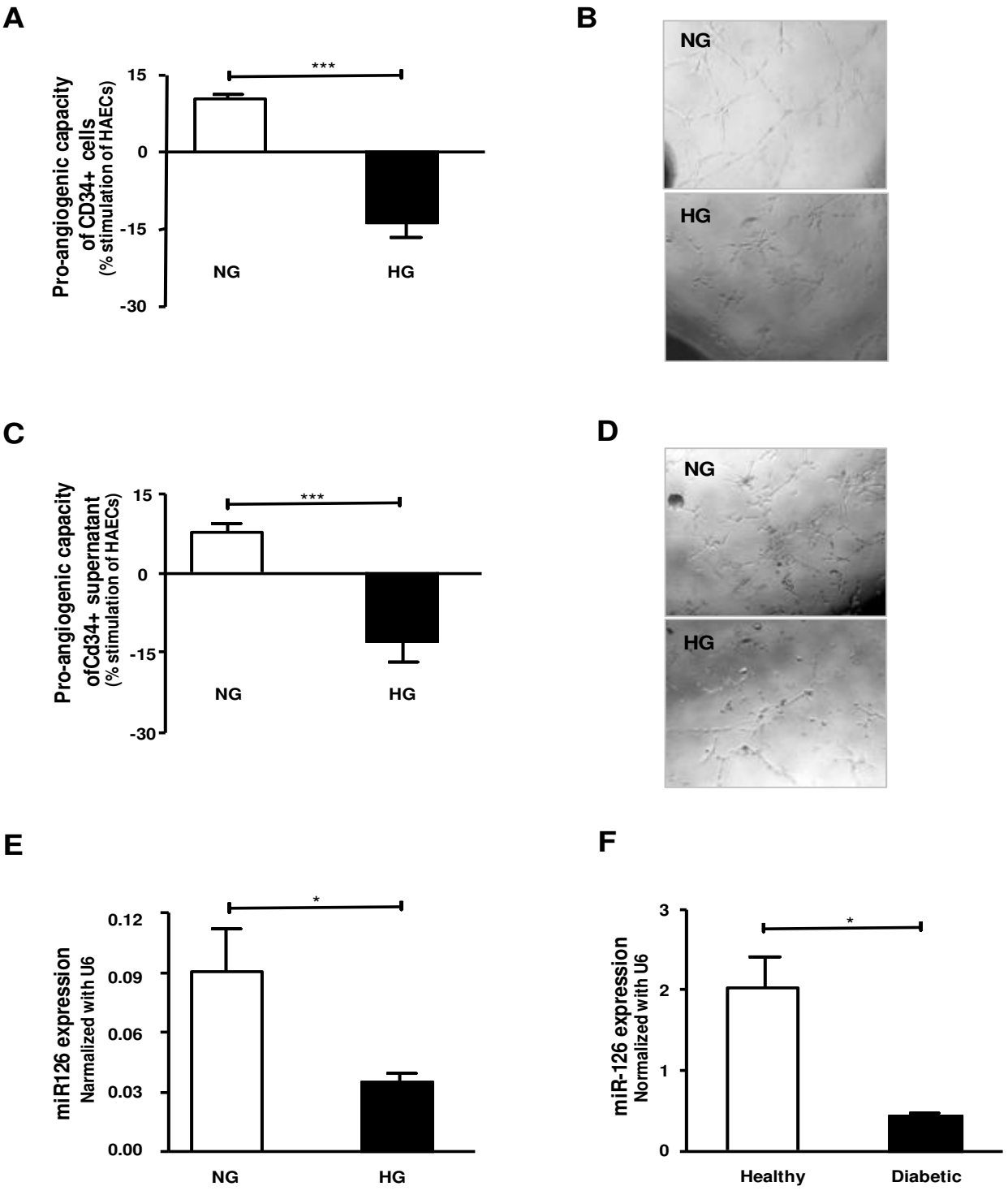
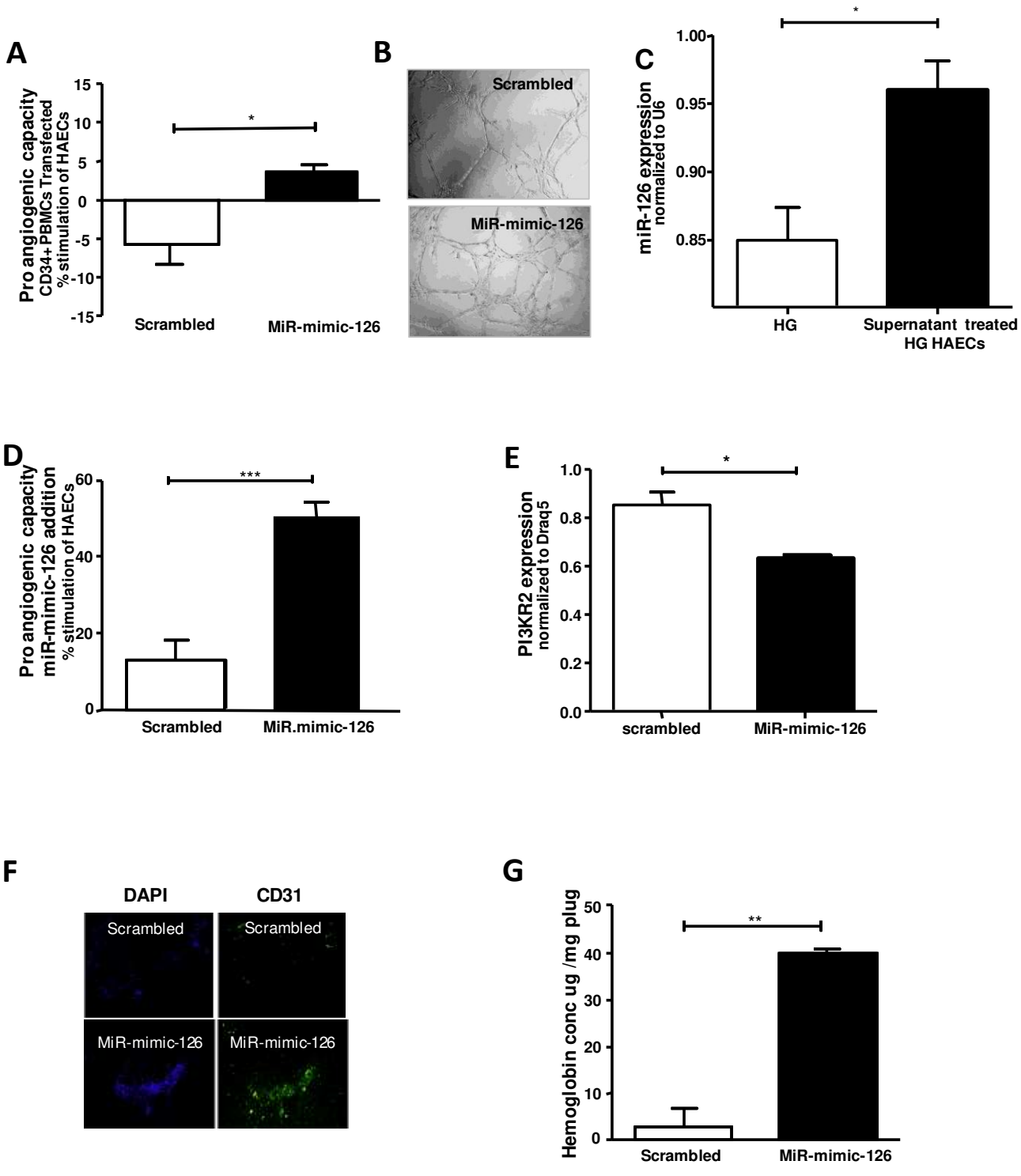

A
Pro-angiogenic capacity of CD34+ cells (% stimulation of HAECs)
15
0
-15
-30
***
NG
HG
B
NG
HG
C
Pro-angiogenic capacity ofCd34+ supernatant (% stimulation of HAECs)
15
0
-15
-30
***
NG
HG
D
NG
HG
E
miR126 expression Narmalized with U6
0.12
0.09
0.06
0.03
0.00
*
NG
HG
F
miR-126 expression Normalized with U6
3
2
1
0
*
Healthy
Diabetic

Figure : 7


A
Pro angiogenic capacity
CD34+ PBMCs Transfected
% stimulation of HAECs
15
10
5
0
-5
-10
-15
*
Scrambled
MiR-mimic-126
B
Scrambled
MiR-mimic-126
C
miR-126 expression
normalized to U6
1.00
0.95
0.90
0.85
*
HG
Supernatant treated
HG HAECs
D
Pro angiogenic capacity
miR-mimic-126 addition
% stimulation of HAECs
60
40
20
0
***
Scrambled
MiR.mimic-126
E
PI3KR2 expression
normalized to Draq5
1.0
0.8
0.6
0.4
0.2
0.0
*
scrambled
MiR-mimic-126
F
DAPI
CD31
Scrambled
Scrambled
MiR-mimic-126
MiR-mimic-126
G
Hemoglobin conc ug /mg plug
50
40
30
20
10
0
**
Scrambled
MiR-mimic-126